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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal addresses the treatment of segmental bone defects and methods that can be used to manipulate the Masquelet induced membrane (IM) to create a graft bed that optimizes bone regeneration. The Chronic Caprine Tibial Defect (CCTD) model is used 1) to improve surgical technique and 2) to optimize spacer design; these are likely to advance bone regeneration strategies and translate into clinical practice

15. SUBJECT TERMS

Optimization of the Masquelet induced membrane to improve bone healing

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1. INTRODUCTION:

Despite substantial advances in the availability of bone graft substitute materials in recent years, large bone defects and chronic bone defects represent a large, and as yet unsolved, clinical challenge. Bone defects are of particular relevance to the recovery and function of injured warriors [1]. This research addresses the treatment of segmental bone defects and methods that can be used to optimize the Masquelet induced membrane [2-6] technique to create a superior graft bed for bone regeneration. Using the Chronic Caprine Tibial Defect (CCTD) model, this study specifically aims to assess the effects of surgical technique and spacer design in optimizing the biology of the "Induced Membrane" (IM), and to define the characteristics of an optimal membrane with respect to the parameters of cell composition, histology, and gene expression. Optimizing the Masquelet IM method and characterization of the biological features of the IM has significant potential to enhance the clinical care of wounded warriors who require bone regeneration procedures and to guide the ongoing development of advanced methods for bone regeneration. This program includes a nested development program for Dr. Jean-Claude D'Alleyrand, a junior orthopaedic surgeon at the Walter Reed National Military Medical Center, to nurture his interest in translational and clinical research by fostering relationships with established, independent clinician scientists with proven track records. The specific aim of this proposal for year 1 is to test the hypothesis that removal of the thin glistening inner surface of the IM created around a smooth polymethylmethacrylate (PMMA) spacer will enhance bone regeneration. Our plan is to characterize the histological, biochemical, cellular and gene expression features of the IM and define the features that best predict the magnitude of bone regeneration following an ACBG.

The broad objective of our team is to accelerate the rate at which clinically significant questions related to surgical management of the "induced membrane", advanced spacer design, and advanced bone regeneration strategies can be translated into clinical practice to improve the care of wounded warriors and civilians.

2. KEYWORDS:
segmental bone defect, caprine tibia, animal model, chronic, bone graft, Masquelet induced membrane, bone regeneration

3. OVERALL PROJECT SUMMARY:

1) Method

16 goats were used in Aim 1. All animals had muscle excised and a smooth spacer placed at the time of the "Pre-Procedure". Autogenous Cancellous Bone Graft (ACBG harvested from the sternum at the time of the treatment surgery is used as the graft material in all animals.

Group 1 – ACBG placed directly into the IM i.e. "not scraped IM"

Group 2 – ACBG placed into the IM after scraping away the inner surface i.e. "scraped IM"

Surgical Protocol

Each animal undergoes two surgeries defined here as: 1) the "pre-procedure" to create the tibia defect and the IM and 2) the "treatment" (4 weeks after "pre-procedure") ACBG) is placed into the IM that is scraped for half of the goats and not scraped in the other half.

The "Pre-Procedure" (Figure 1) is comprised of the following essential features:
1. Make a medial skin incision and excise a 5-cm segment of tibial diaphysis and periosteum.
2. Excise an additional 2 cm of periosteum on the proximal and distal bone segments.
3. Debride 10 grams of tibialis anterior and gastrocnemius muscles.
4. Place an interlocking intramedullary nail using a custom spacer to maintain 5-cm defect length.
5. Place a pre-molded 5 cm long x 2 cm diameter PMMA spacer around the nail in the defect.
6. Irrigate the wound with normal (0.9 %) saline and wound closure.

The "Treatment" (Figure 2) is performed 4 weeks after the "Pre-Procedure" is comprised of:

1. Collect ACBG from sternebrae (Figure 3). 12 cc of cancellous graft is needed for each defect; all graft available is collected from sterrnebra 4 or 5, approaching other sternebra only as needed.
2. Open the previous skin incision on the medial aspect of the tibia.
3. Open the IM surrounding the PMMA spacer using a "bomb bay door opening".
4. Remove the spacer without damaging the membrane or nail.
5. Collect appropriate IM samples as defined below (see section b).
6. In Aim 1, the inner layer of the IM was scraped away in one half of the goats before grafting and the graft was placed in the intact IM in the remaining goats.
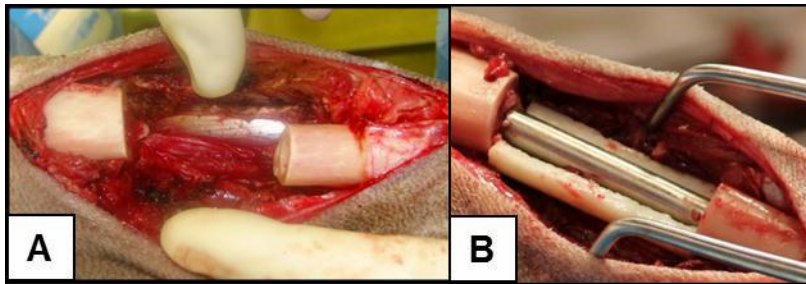7. Close the IM with 3-0 nylon to provide an intrinsic marker and close the remaining tissues.

Figure 1 - "Pre-procedure" surgery images. A) After excision of 5 cm tibia, 9 cm periosteum, 10 gm muscle and intramedullary reaming. B) After placement of interlocking IM nail and half of the PMMA spacer.
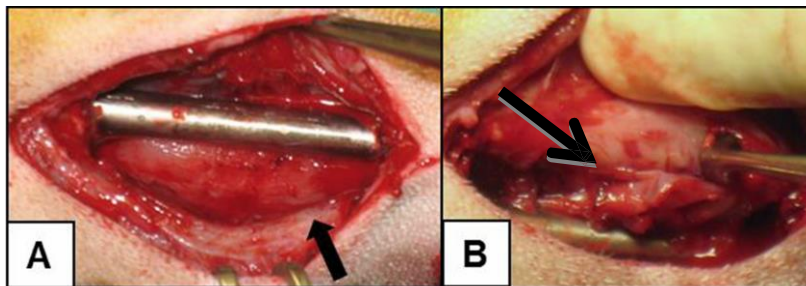

Figure 2 - "Treatment" surgery images.**pictures.** A) Four-week-old defect, after spacer removal. Black arrow shows the thick-walled (~4mm) IM and the thin friable inner layer **at "treatment" surgery.** B) Scraping the thin inner surface of IM prior to grafting. The scraped layer can be seen overlying the nail in the defect space (black arrow).
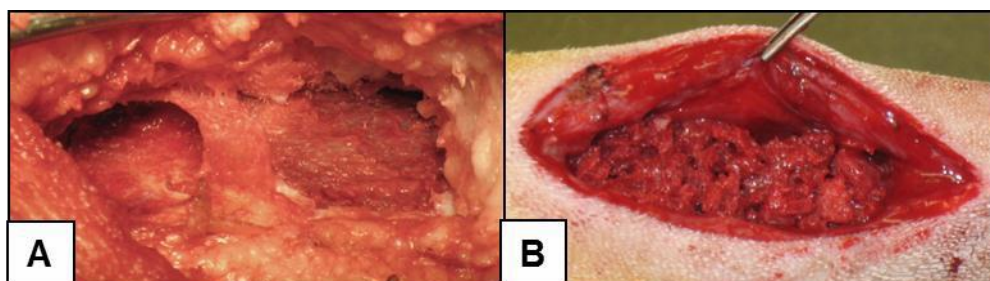

Figure 3 - Collection of ACBG from the goat sternum. A) Two sternebrae with outer cortex removed and cancellous graft harvested (mean 7 cc/sternebra). B) ACBG placed into the defect site.

The procedures after treatment include:

– Orthogonal radiographs (anterior-posterior (AP) and mediolateral (ML) projections) of tibias every 4 weeks
– Physical examination including lameness grading daily, then biweekly starting 2 weeks after "Treatment" surgery.

Euthanasia is 12 weeks after "Treatment" surgery at which time tibias are harvested and fixed in 10% formalin. Micro CT & histologic analyses of regenerate tissue are then performed.

Induced membrane collection protocol during "Treatment" surgery

By designing the spacer to create an IM that has a diameter 7 mm larger than the tibial diaphysis, 7 mm of IM can be harvested at the time of spacer removal, prior to grafting, as shown in Figure 4. Figure 4 illustrates the methods used to harvest and subsample the IM. Data collected from each sample include weight and thickness. Specific segments are analyzed for cells and osteogenic connective tissue progenitors (CTP-O) ("C"), histology ("H"), and gene expression ("GE"). In the case of "C" and "GE" samples, each segment is divided into an "inner layer" (thin, friable and vascular, immediately adjacent to the spacer) and an "outer" layer (thicker, fibrous, mechanically robust, and less vascular.

Four samples are minced and digested in collagenase I and dispase for cell and CTP-O analysis. An assay for CTP-O is performed using established ASTM standard methods [7].

Samples H2 and H7 are fixed in 10% neutral buffered formalin (NBF) and transferred to 70% ethanol 48 hours later, then shipped to the laboratory of Dr. Carlson at the University of Minnesota for processing into paraffin blocks. 4-µm thick sections from each bloc, stained with H&E and Masson's trichrome, are examined by Dr. Carlson and imaged at low and high magnifications. A 1-cm^3 sample from each ACBG sample is characterized by histomorphometry to define areas of hematopoietic marrow, bone, adipose tissue, vascular spaces, fibrous tissue, and void spaces.

Samples GE3 and GE6 are stored in RNAlater and shipped to Dr. Davis at the NMRC for gene expression analysis.
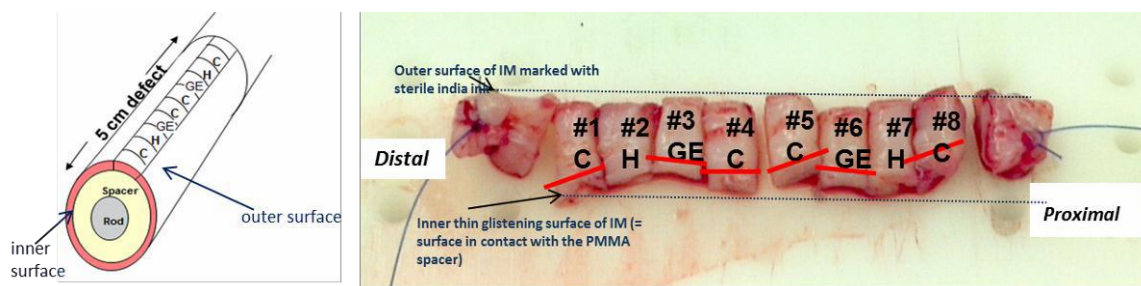

Figure 4 - 5cm **Induced Membrane as 7 mm** x 7mm strip of induced membrane. Each strip of IM is sectioned into inner and outer portions for analysis of cells and gene expression **(GE3, GE6).** The histology sections are kept intact.

2) Results
   a) Surgeries (Cleveland Clinic – Drs. Muschler, Pluhar and D'Alleyrand)
   Surgery was performed on 16 goats at Cleveland Clinic by the surgical team consisting of Drs. Muschler, Pluhar and D'Alleyrand. Each goat had the pre-procedure to create the segmental defect and the subsequent treatment surgery four weeks later. We experienced one complication during anesthetic recovery from the treatment surgery that led to the animal death and removal from the study.

   b) Radiographic Assessment (Cleveland Clinic – Dr. Muschler)
   Orthogonal radiographs (anterior-posterior (AP) and mediolateral (ML) projections) of all 15 goats were made every 4 weeks (see **Figure 5**).. The radiographs taken at euthanasia (12 weeks) are shown in **Figure 6**. The radiographs demonstrate variable amounts of bone formation from both the unscraped (not scraped) and scraped IM goats.
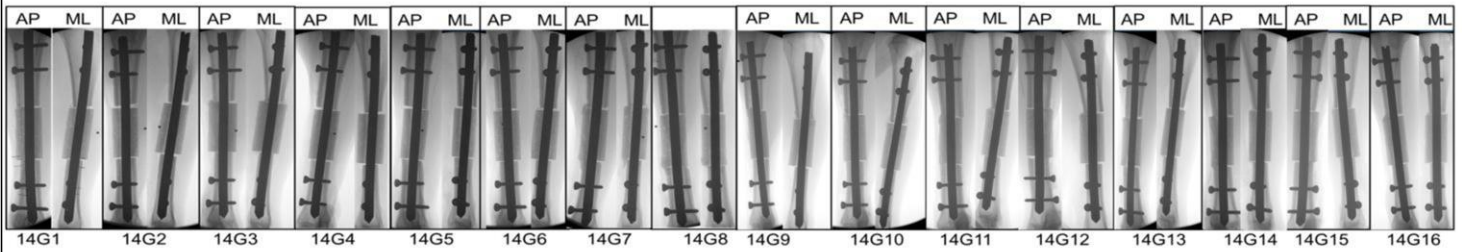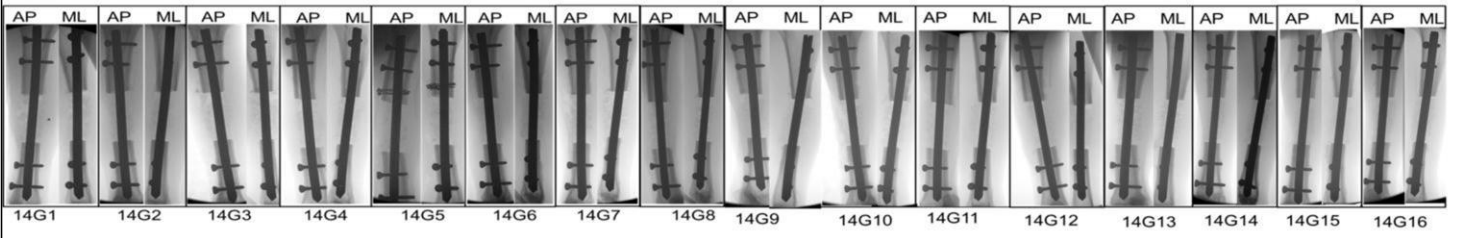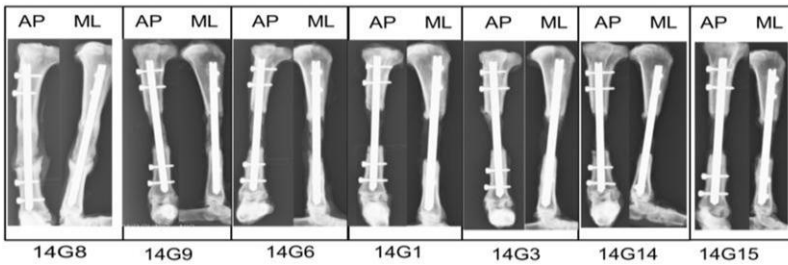
Figure 5 - Radiographs taken at A) pre-procedure surgery showing placement of the spacer in the defect site; B) treatment surgery where the ACBG is placed in the induced membrane with or without scraping.
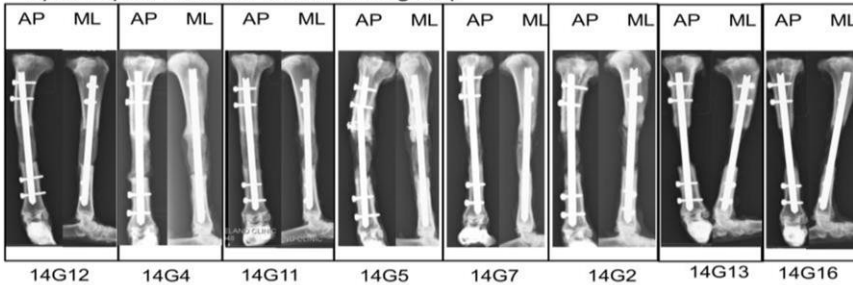

Figure 6 - Radiographs taken 12 weeks after grafting with A) unscraped IM; B) scraped IM.

c) MicroCT analysis (Cleveland Clinic - Dr. Muschler)
The primary outcome of bone formation was assessed by microCT. These data are graphically presented using a heat map in Figure 7. The plots (mean percent bone volume (%BV)) in Figure 7A illustrate that there is greater bone formation (>60%) near the osteotomies in both groups. The mean %BV plot for the unscraped IM group ranged between 2% to 25%, and for the scraped IM group ranged between 30 to 40 %. This suggests that scraping the IM tends to induce greater bone formation with less variability. The angular moment plots in Figure 7B illustrate that bone tended to form along the posterior aspect of the defect for the unscraped IM group with less bone formation medially and laterally. In the scraped IM group, more bone tended to form on the posteriorpreferentially formed posteriorly and lateral aspects with little bone formation medially. There was a trend for greater bone formation in the scraped induced membrane group. Statistical analysis in the next quarter will determine if this increase is significant.
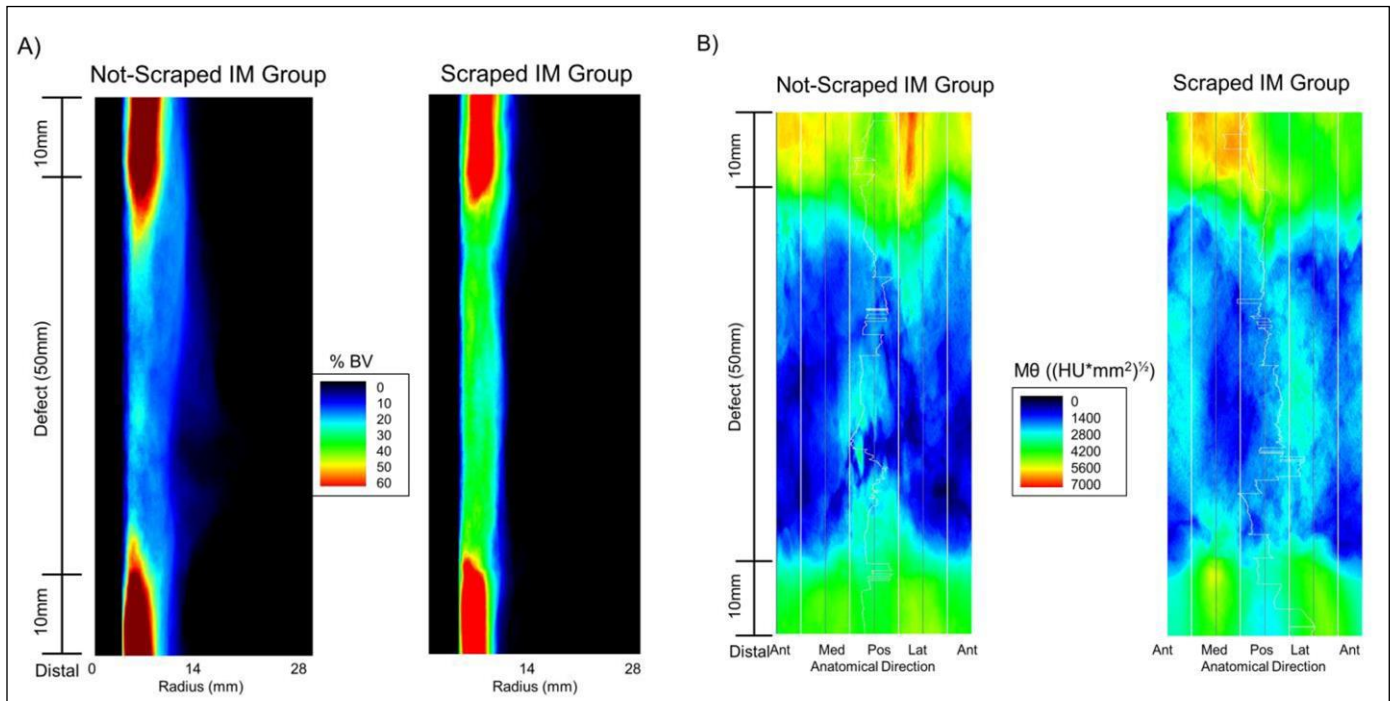
Figure 7 - Plots created from microCT data demonstrating the effect of scraping the IM: A) mean percent bone volume plots the radial position in the x-axis and length of the tibia in the y-axis for each treatment group; B) mean angular moment plots the radial position in the x-axis and length of the tibia in the y-axis for each treatment group

d) Histology analysis (University of Minnesota - Dr. Carlson)
   The following sectioning, staining, and processing of these samples have been completed:
   - Induced membrane histology (N=32; 2 samples x 16 goats). Two samples H2 and H7 were received from each of 16 goats (goats #14G1-14G16) and samples of scraped inner layer were received from 3 of these goats (goats 14G12, 14G13, and 14G16). All were processed for histology, stained with H&E and Masson's trichrome, examined by a Dr. Carlson, and imaged using 1X and 10X objectives.
   - ACBG samples (N= 16; 1 sample x 16 goats). A/16 samples). One sample of ACBG collected from the sternum, was received from each of 16 goats (goats #14G1-14G16, excluding goat #14G10). Each was processed for histology, stained with H&E, examined by Dr. Carlson, and imaged using 1X and 10X objectives.
   - Tibiae (15 samples). The treated tibia from each of 15 goats (previously fixed in 10% NBF) was received. All tibiae were radiographed and placed in decalcification solution. Sectioning and staining will be performed in the next quarter.

Histological examination of the H&E stained induced membrane reveals a relatively bland but consistent histological appearance. Each IM has a narrow inner later or zone (adjacent to the PMMA) composed of mononuclear cells that appear to be primarily macrophages, but likely other cell types as well. An occasional multinucleated giant cell is present in this zone. Subjacent to this narrow zone, the tissue is composed of granulation tissue that extends to the outermost margin of the section. All samples exhibit extensive, diffuse trichrome positivity indicative of the presence of fibrous connective tissue. Samples of the scraped inner layer of the induced membrane (collected at the time of the "pre-procedure" surgery) have a similar appearance to the inner zone of the induced membranes (whether from scraped or control groups) that were examined at the time of necropsy/tissue collection. Immunostaining studies are pending on these samples to confirm the presence of specific proteins and to characterize their distribution within the membrane depending on the results of biochemistry and/or gene expression results.

All sections from the ACBG samples contain abundant bone trabeculae and bone marrow; bone marrow is moderately cellular in all sections. Very minimal extraneous tissue, usually composed of small foci of hyaline cartilage, is present in some of the sections. All samples are subjectively assessed as being of high quality graft material.
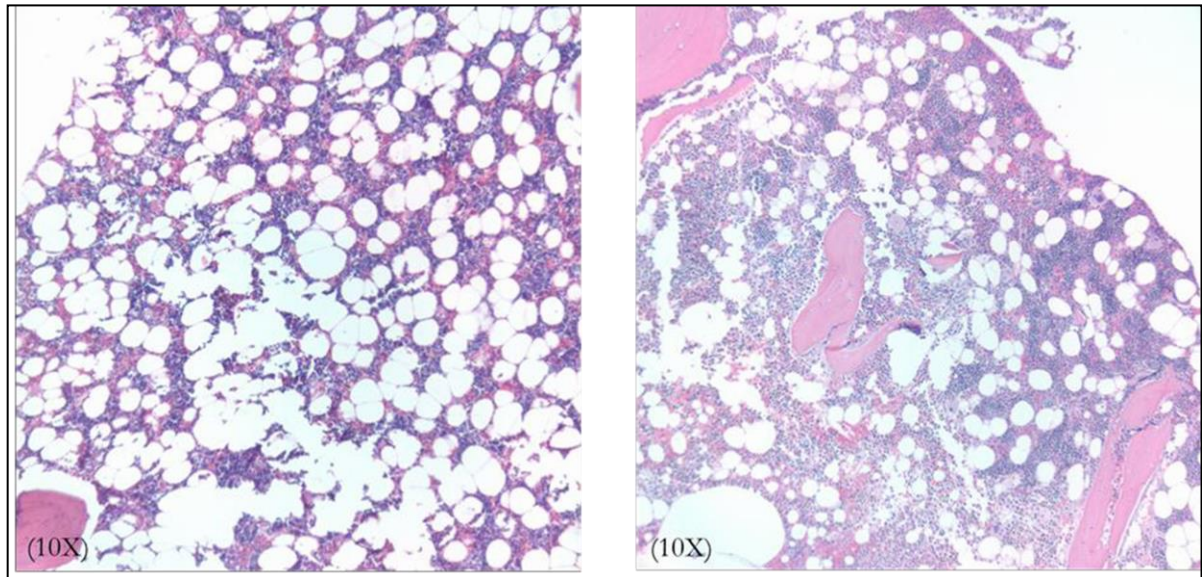

Figure 8 - Representative histology examples of sections of ACBG graft material demonstrating foci of cancellous bone accompanied by abundant hematopoietic cells (10X; H&E).
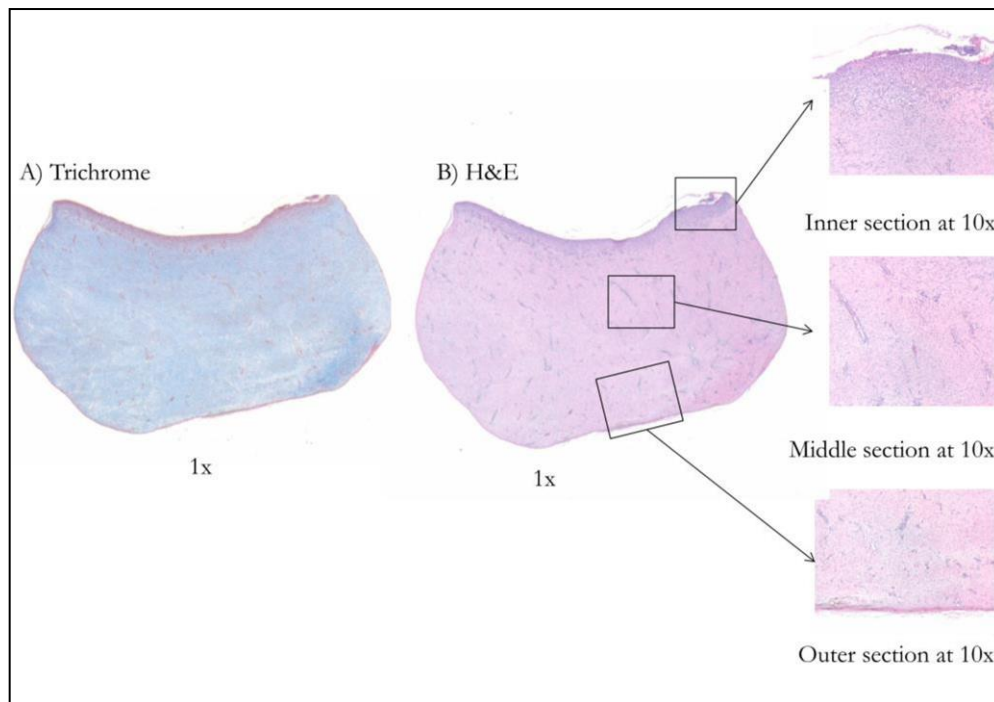

Figure 9 - Representative histology section of IM stained with A) Masson's trichrome demonstrating diffuse positivity indicating the presence of fibrous connective tissue and B) H&E showing narrow inner zone (adjacent to the PMMA) composed of mononuclear cells that appear to be primarily macrophages.

e) Characterization of the concentration, prevalence, and biological performance of ACBG and IM (Cleveland Clinic – Dr. Muschler)

Concentration and prevalence of CTP-Os were assayed using the "C" pieces of the IM using established methods for mincing, dispase-collagenase digestion, and cell counting. The cellularity of the IM and prevalence of CTP-Os were assessed in the 16 goats. A mean of 18,539 +/- 17,590 million cells were found per milligram weight of IM sample. The cellularity tended to be higher

in the inner IM than the outer IM, respectively 25,582+/- 22,183 million cells per mg for the inner IM and 11,495+/- 12,998 million cells per mg for the outer membrane (Figure 10). Evaluation of initial plating densities from inner and outer IM tissue samples was completed to enable determination of an optimum range of plating density for colony forming unit (CFU) analysis at day 6 harvest. CTP Prevalence revealed to be very high which did not allow counting colonies. The plating density in the next experiment will need to be reduced by five times at the next experiment. A lower plating density at 20,000 cells per chamber has now been selected for colony analysis.
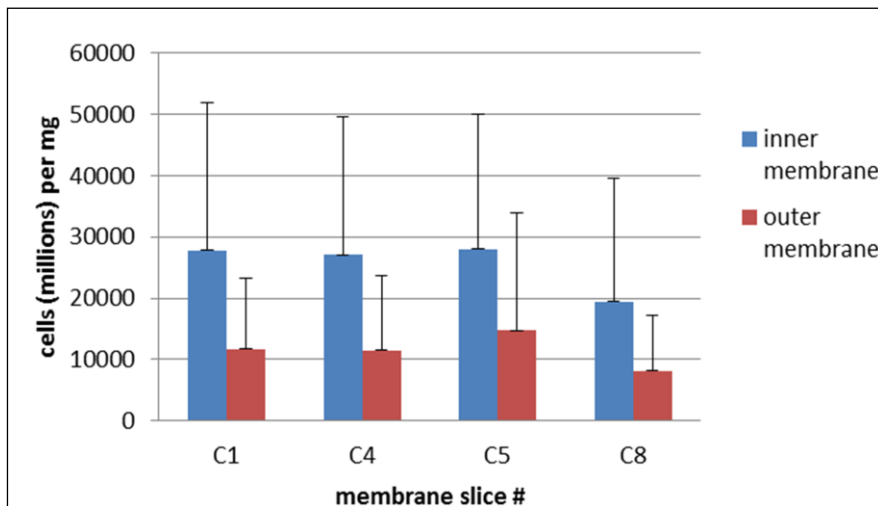

Figure 10 – Cell counts for each inner and outer **membrane** slices

3) Gene expression analysis (Naval Medical Research Center - Dr. Davis)

Goat induced membrane samples were shipped from Cleveland Clinic to NMRC in RNAlater. RNA extraction was done for all samples from 50 mg of tissue using the RNeasy Plus Universal Kit (Qiagen), and quality control was done via Nanodrop and Agilent. RNA samples were converted to cDNA via the SaBioscience cDNA conversion kit. Initially gene expression analysis was performed using SaBioscience custom human osteogenic arrays on two goat samples. Results were inconclusive as Ct values were above the threshold or undetectable. The sequences of human gene primers used were compared to goat gene sequences (http://www.ncbi.nlm.nih.gov) and the homology was found to be low, requiring the design of custom primers for analysis of goat gene expression).

A panel of genes was selected (see Table 1) and primers were designed specifically for goat. The nucleotide sequences of all candidate genes (Table 1) belonging to the domestic goat (*Capra hircus*) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). Primer pairs were designed from these sequences (optimal T_m at 60°C and GC% between 45-50%) using primer3Plus online software and checked using OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/), Beacon Designer (http://free.premierbiosoft.com) and PrimerBLAST. Primers were ordered from Life Technologies.

RNA for all samples was converted into cDNA using the SuperScriptIII First-Strand Synthesis SuperMix kit (Life Technologies, # 18080-400). A primer optimization was necessary to optimize the concentration of primers to be used to run real-time PCR. Initially a primer optimization was done per the Power SYBR Green PCR Master Mix protocol in order to optimize the concentration of primers to be used that will give the lowest Ct. Several primer concentrations were tested to determine the ideal concentration that would produce the best gene expression results. The ideal concentration was found, and will be used to complete a gene expression analysis of all tissue samples for the genes specified.

Gene	Function
RUNX2	Essential for osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression.
OSTERIX	Bone specific transcription factor that is required for osteoblast differentiation and bone formation.
PPARG	The protein encoded by this gene is PPAR-gamma and is a regulator of adipocyte differentiation.
OCT4	Encodes a transcription factor containing a POU homeodomain that plays a key role in embryonic development and stem cell pluripotency.
SOX2	Encodes a member of the SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate.
NANOG	Transcription factor involved in maintaining pluripotency in embryonic stem cells.
ALPL	Encodes a membrane bound glycosylated enzyme, a proposed function of this form of the enzyme is matrix mineralization.
EGFR	Encodes a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family.
VWF	Encodes a glycoprotein which functions as an antihemophilic factor carrier and a platelet-vessel wall mediator in the blood coagulation system. It is crucial to the hemostasis process.
PDGFB	Protein encoded by this gene is a member of the platelet-derived growth factor family.
BMP2	Encodes a protein that acts as a disulfide-linked homodimer and induces bone and cartilage formation.
BMP6	Part of a family of secreted signaling molecules that can induce ectopic bone growth. This gene has a proposed role in early bone development.
COL1A1	Encodes a fibril-forming collagen that is found in most connective tissues and is abundant in bone, cornea, dermis and tendon.
COL2A1	Encodes the alpha-1 chain of type II collagen, a fibrillar collagen found in cartilage.
COL10A1	Encodes the alpha chain of type X collagen, a short chain collagen expressed by hypertrophic chondrocytes during endochondral ossification.
GLA	Encodes a homodimeric glycoprotein that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins.
IBSP	Protein encoded by this gene is a major structural protein of the bone matrix
PTHR	A member of the G-protein coupled receptor family 2 that is a receptor for parathyroid hormone (PTH) and for parathyroid hormone-like hormone (PTHLH).
EGF-A	Is a glycosylated mitogen that acts on endothelial cells and has various effects, including mediating increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis

Table 1. Selected panel of genes and primers designed specifically for goat

4) Predictive modeling (Walter Reed National Military Medical Center - Dr. Forsberg)
   Development of database resource for collection and management of data from above sources in Aim 1 and construction of the data shell are in progress and will be ready to be populated by the data collected in Aim 1 in 2-3 months.

5) Nested development award (Walter Reed National Military Medical Center - Dr. D'Alleyrand)
   Dr. D'Alleyrand has had the opportunity to interact with Drs. Pluhar and Muschler and the veterinary staff at Cleveland Clinic to become engaged and knowledgeable of the animal care process, and he has participated in group laboratory discussions. He has been oriented to the processes of cell, CTP, histology, and microCT analysis. In the current phase of work he will receive more specialized training and orientation to microCT analysis, both on a theoretical and practical level. He will work with raw CT images and gain an operating experience with the process of moving from raw images to quantitative data, including sources of variation and error and will interact directly with the biostatistics team during the integration and analysis process. He will participate intimately in the

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process of preparing summary data, presentation materials, giving presentations in selective settings (e.g. OTA, SOMOS), and contribute as an author and editor for manuscript submissions.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

- Performed surgeries on 16 goats with 12 weeks of radiographic follow-up and microCT analysis demonstrating the scraping of the induced membrane tends to increase new bone formation. Statistical of the total bone volume calculated from the MicroCT data will be performed in the next quarter to assess if this increase in new bone is statistically significant.
- Performed histology on induced membrane samples that illustrated the presence of a narrow inner zone (adjacent to the PMMA) composed of mononuclear cells and exhibited extensive, diffuse fibrous connective tissue.
- Performed histology on goat autogenous bone graft which demonstrated that the quantity and quality of cancellous bone graft and bone marrow aspirate from the sternum has great cellularity
- Performed cell/CTP analysis on induced membrane samples that showed CTP Prevalence was very high which did not allow colony count. The plating density in the next experiment will need to be reduced by five times at the next experiment. A lower plating density at 20,000 cells per chamber has now been selected for colony analysis.
- Performed gene expression on induced membrane that led to selection of a panel of genes and creation of custom-made goat primers.

5. **CONCLUSION:**

Year 1 is assessing the value of surgically removing the thin region of the foreign body response, as a means of changing the local cell population and tissue surface to which a graft material is exposed, including the vascular plexus beneath the thin inner membrane.

Statistical analysis is ongoing. However, radiographic and microCT images suggest that scraping of the IM results in an increase in bone formation in defect sites that were grafted using ACBG. Scraping the IM layer seems to provide a highly desirable environment of growth factors and cytokines, providing exposure of the ACBG placed in the defect site to fresh blood and competing non-osteogenic cells that may promote bone regeneration. Scraping away the thin surface layer of the IM might remove an undesirable barrier and enhance bone regeneration.

Goat Surgeries for Year 2 of this study will begin in January, addressing the question of macroporosity in PMMA antibiotic delivering spacers.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**
   a. Publication: Nothing to report.
   b. Presentations:
      Pluhar E., Luangphakdy V., Boehm C., Shinohara K., Pan H., Carlson C., Muschler G.F.: Effect of Grafting : Materials on Bone Healing in a Chronic Caprine Tibial Defect Model, 2014 Military Health System Research Symposium (MHSRS). August 18-21, 2014.

7. **INVENTIONS, PATENTS AND LICENSES:**
   Nothing to report

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8. REPORTABLE OUTCOMES:"

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9. OTHER ACHIEVEMENTS:"

Pqyjkpi "vq'tgrqtv'

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10. REFERENCES:"

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2. Xkcvgcw. "X0"gv'cn0 "*Induction of a barrier membrane to facilitate reconstruction of massive segmental diaphyseal bone defects: an ovine model. Xgv'Uwti . "4228035*7+<r0667/740'

3. Ocuswgngv. "CŒ0"gv'cn0 "[Reconstruction of the long bones by the induced membrane and spongy autograft]. Cpp'Ejkt'Rncuv'Guvjgv. "4222045*5+<r0568/750'

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11. APPENDICES: n/a

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